

AD _____

Award Number: DAMD17-03-1-0201

TITLE: Arginase: A Novel Proliferative Determinant in Prostate Cancer

PRINCIPAL INVESTIGATOR: Wayne W. Grody, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of California
Los Angeles, CA 90024

REPORT DATE: April 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20041123 063

BEST AVAILABLE COPY

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE April 2004	3. REPORT TYPE AND DATES COVERED Annual (1 Apr 2003 - 31 Mar 2004)	
4. TITLE AND SUBTITLE Arginase: A Novel Proliferative Determinant in Prostate Cancer			5. FUNDING NUMBERS DAMD17-03-1-0201	
6. AUTHOR(S) Wayne W. Grody, M.D., Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California Los Angeles, CA 90024 E-Mail: wgrody@mednet.ucla.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) This project is an investigation of the involvement of the enzyme arginase type II (AII) in the pathogenesis and growth of prostate cancer. Having recently cloned the AII gene in our laboratory, we unexpectedly discovered that it is expressed at high levels in the normal prostate and even higher in neoplastic prostate samples. The purpose of the present research funded by USAMRMC is to examine the expression of AII in a wider range of benign and malignant prostate specimens and cultured cells to determine its usefulness as a novel marker of prostatic neoplasia and the extent of its involvement in cancer pathogenesis. We will also explore whether specific chemical and molecular inhibitors of arginase and several related enzymes in the polyamine metabolic pathway might suppress or arrest the growth of prostate cancer cells <i>in vitro</i> or <i>in vivo</i> . This first annual report describes our progress to date in developing the overexpression and inhibitory vector constructs needed for the research, our initial optimization of gene transfer techniques in prostatic cancer cells, and characterization of arginase and related enzymes in prostate cancer lines of various degrees of differentiation.				
14. SUBJECT TERMS Prostate cancer, arginase, polyamines			15. NUMBER OF PAGES 25	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	21
Reportable Outcomes.....	22
Conclusions.....	23
References.....	24
Appendices.....	N/A

Introduction

Prostate cancer is the most common and second most lethal cancer among men in the United States, yet despite its high prevalence, relatively little is known about the biochemical and molecular mechanisms controlling benign and malignant prostatic growth. We have proposed to investigate the involvement of the enzyme arginase type II (AII) in this malignancy. We believe that this enzyme plays a pivotal role in the synthesis of polyamines, chemicals involved in cell growth and regulation that are found in high levels in normal prostate tissue and in cancer cells. Having recently cloned the AII gene in our laboratory, we unexpectedly discovered that it is expressed at high levels in the normal prostate and even higher in neoplastic prostate samples. The purpose of the present research funded by USAMRMC is to examine the expression of AII in a wider range of benign and malignant prostate specimens and cultured cells to determine its usefulness as a novel marker of prostatic neoplasia and the extent of its involvement in cancer pathogenesis. We will also explore whether specific chemical and molecular inhibitors of arginase and several related enzymes in the polyamine metabolic pathway might suppress or arrest the growth of prostate cancer cells *in vitro* or *in vivo*. The specific aims of the project are to determine: (1) the specific prostate cell types responsible for the high-level expression of arginase AII, (2) the role of arginase in critical pathways of polyamine and nitric oxide synthesis in benign and malignant prostatic growth, (3) the mechanism and efficacy of targeted molecular and biochemical inhibitors of the arginase pathway in blocking the growth of prostate cancer cells, (4) the effect of genetically engineered overexpression of arginase and related enzymes on prostate cancer cell growth, and (5) the significance of arginase AII activity as a potential novel diagnostic marker and/or therapeutic target of prostatic neoplasia *in vivo*. We believe this work may shed light on the fundamental mechanisms of prostatic neoplasia while at the same time suggesting new directions for diagnosis and therapeutic intervention.

Body of Report

Following a longstanding interest in the first discovered liver isoform of arginase (AI) as the focus of a rare inborn error of metabolism (arginase deficiency; hyperargininemia), our laboratory more recently has begun to focus on the role of the second, extrahepatic isoform, arginase II (AII) and its potential as a novel marker for prostate cancer. Arginase II is highly expressed in the normal prostate and even more highly expressed in patients with prostate cancer. A major overarching goal of the funded project is to artificially engineer cells, tissues, and model organisms (mice) to achieve overexpression or inhibition of the arginase isozymes and other related genes and enzymes of arginine metabolism and study the effect on prostate cancer cell growth. Much of the initial year of the project has therefore been spent developing and testing the necessary reagents for manipulating these genes in the various target milieus specified in the grant proposal. While not entirely completed yet, we feel we have thus laid the groundwork for the proposed experiments to follow. A summary of these accomplishments follows, with reference made to the specific items in the approved Statement of Work to which they apply.

The key genes we have chosen to focus on, by virtue of our postulated involvement of them in prostate cancer growth, are arginases I and II, ornithine decarboxylase (ODC), agmatinase, ornithine aminotransferase (OAT), and arginine decarboxylase (ADC). Each of these enzymes functions within the extended arginine metabolic pathway (Fig. 1), which our group, primarily in a companion grant from the

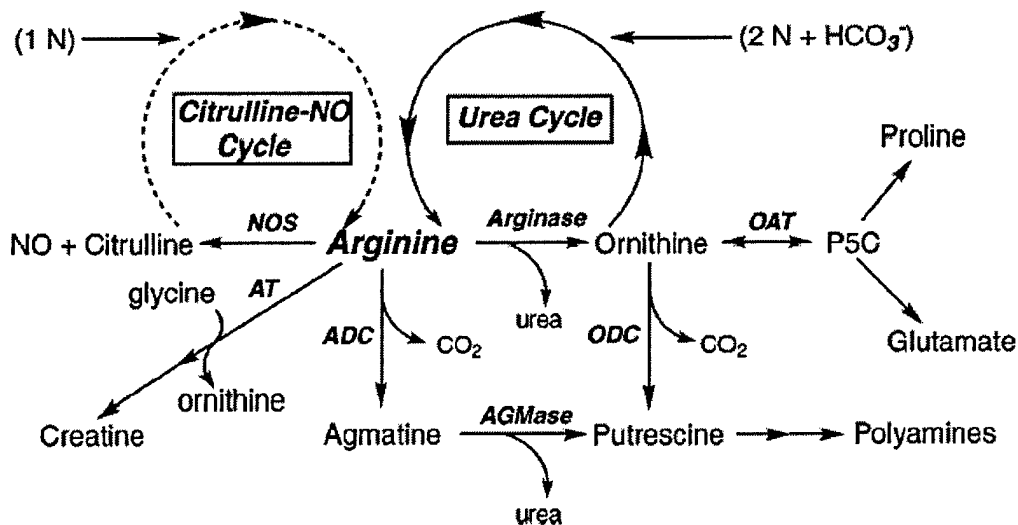


Figure 1. The arginine metabolic pathway, showing side reactions toward polyamine synthesis (Morris, 2002).

NIH headed by Dr. Stephen Cederbaum, has been working out. During this first year we have constructed overexpression elements containing GFP (green fluorescent protein)

fusions under the control of the CMV constitutive promoter for each of the above genes, and have tested them using a variety of enzymatic assays. In addition, interfering RNA (siRNA) constructs (Lieberman *et al.* 2003) targeting all of the above genes have been created and validated using RT-PCR. These reagents are all essential for completion of Tasks 3 and 4.

Task 1.a Arginase Isoforms in Prostatic Tissue and Cell Lines

For use as crucial target milieus for these studies, we have established and presently have growing in culture several prostate cell lines, and we are in the process of creating stably transfected cell lines for several of the above constructs. The cell lines currently being utilized include LNCaP, an androgen receptor-positive metastatic prostate carcinoma line, as well as DU 145 and PC-3, both of which are androgen receptor-negative. We are also growing PZ-HPV-7, an immortalized cell line representing essentially normal prostate.

Initially we have decided to analyze these cell lines using semiquantitative RT-PCR and immunoprecipitation (Figure 2; Figure 3), techniques that we had previously worked out for studies of our arginase knockout mouse (for technical conditions, see Yu *et al.* 2003). We have designed and validated PCR primers for all of the various genes and will be using them to analyze changes in expression resulting from both transient and stable transfections of our overexpression constructs and siRNAs. As can be seen in Figure 4, there are definite differences between the various prostatic cell lines in the degree of gene expression. These results are taken from the native cell lines without any alterations. It may be noteworthy that the most differentiated cell line, namely LNCaP, has the highest level of AII expression and seems to be the only one with any AI expression.

Furthermore, it may also be significant that as the prostate cell lines become less well differentiated and androgen-independent, besides an apparent decrease in the expression of both arginase isoforms there is an increase in the expression of ADC and agmatinase. Previous work had shown that arginase activity was detectable in prostate adenocarcinoma tissue as well as in the serum from patients with metastatic prostate cancer, though expression levels were greater in adenocarcinoma (Table 1). Based on these limited data, elevated arginase activity may be more characteristic of early-stage carcinoma than late-stage metastatic cancer. It is intriguing to speculate about the utilization of an alternate route for polyamine biosynthesis, namely through ADC and agmatinase, in metastatic prostate cancer. Results were unavailable at the time of this submission from the PZ-HPV-7 cell line representing normal human prostate tissue; for now, the expression profile from the human embryonal kidney HEK293 cell line is provided for comparison.

In future experiments, we will be performing Northern and Western blotting as well as direct enzyme assays in order to further characterize these alterations in gene expression.

Table 1. Arginase Activity in Prostate Tissue and Serum Specimens.

<u>Specimen</u>	<u>n</u>	<u>Arginase Activity</u> <u>(ng/mg protein)</u>
Normal prostate	11	12.5 \pm 3.3
Prostate adenocarcinoma	11	64.7 \pm 28.6
Serum from pt. with 1 ^o prost. ca.	5	2.3 \pm 0.3
Serum from pt. with met. prost. ca.	7	29.8 \pm 1.7
Control serum	10	2.1 \pm 0.4

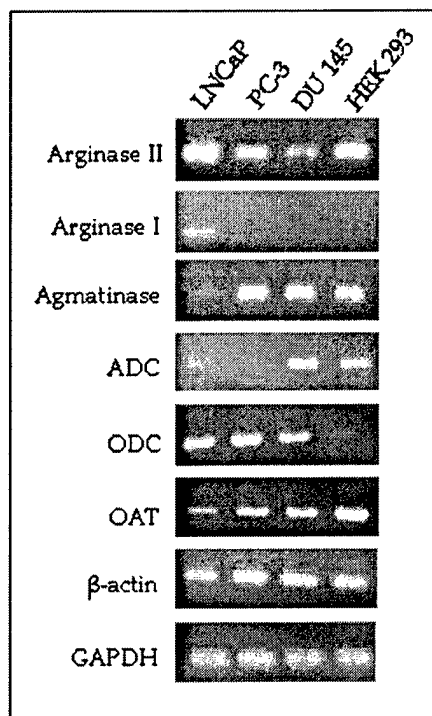


Figure 2. AII, AI, ADC, ODC, and OAT expression in native cell lines by RT-PCR. AII expression was seen most strongly in the LNCaP cell line, whereas AI was seen exclusively in the same line. Agmatinase expression was primarily seen in the less differentiated cell lines, namely PC-3 and DU-145. ADC was most highly expressed in the DU-145 cell line, and ODC was expressed roughly equally in all three prostate cancer cell lines. OAT expression was roughly uniform in all lines except LNCaP. HEK 293 human embryonal kidney cells were used for comparison as a non-prostatic cell line, and expression of β -actin and GAPDH were used as controls for normalization.

Task 1.b In Situ Hybridization and Immunohistochemistry

While the first part of this aim has been addressed by measuring endogenous levels of expression of arginase and related enzymes in our target prostatic cell lines (Figure 2), we have had to defer much of the *in situ* expression studies in cells and tumors because of the unexpected departure of the designated postdoctoral fellow on the project, Dr. Hong Yu. She was our resident technical expert on *in situ* hybridization and immunohistochemistry, which are the essential techniques for accomplishing this Task. We are presently in the process of recruiting a new postdoctoral fellow or technician with this expertise, among others. Figure 3 shows some early immunoprecipitation work that we hope to expand upon once a new team member is hired.

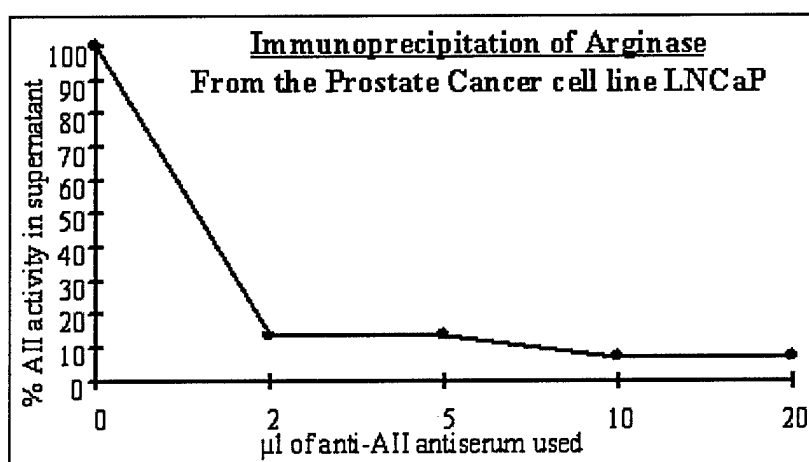


Figure 3. Precipitation of arginase activity in LNCaP cells using anti-AII antibody, demonstrating that almost all of the arginase in this prostatic cancer cell line is AII.

Task 2.a Polyamine Measurements

A mass spectrometry method for analyzing polyamine levels in cell pellets had been developed and has been undergoing further validation through our collaboration with the Pasarrow Spectrometry Laboratory in the Brain Research Institute here at UCLA. This method builds upon previous gas chromatography methods (Dourhout *et al.* 1997), which use flame ionization and nitrogen/phosphorus detection, and instead uses electron capture chemical ionization. Analysis of polyamine standard solutions in the CI negative ionization mode show that the background noise level is reduced and the high m/z ions from each polyamine provide for good specificity.

However, we have found great difficulty in translating this methodology to the analysis of cell lysates and cell culture media. Though many samples have already been analyzed, the data have not been reproducible, and the Micromass GCT time-of-flight mass spectrometer that has been utilized for these studies seems to be inadequate for our use. Therefore, we have decided to seek alternative collaborations for polyamine analysis

and have been in contact with Dr. Guoyao Wu from Texas A&M University as well as Dr. William O'Brien from Baylor College of Medicine regarding this matter. Dr. Wu has agreed to a collaboration and has provided us with protocols for the preparation of cell lysates for polyamine analysis from cultured cells and animal tissues. We are also looking into a collaboration with Dr. O'Brien due to his personal history with our group and the potential for a more cost-effective service.

Task 2.b Polyamine Synthetic Enzyme Analysis

As shown previously (Figure 2), we have already begun work on this task using semiquantitative RT-PCR and enzymatic assays and will be further expanding on such data using Northern and Western blotting. Data is provided below showing arginase enzymatic activity assayed using our standard protocol based on the cleavage of [14 C]arginine (Spector *et al.* 1980).

LNCaP arginase activity is compared with that of two human liver cell lines, H2-35 and HepG2, human embryonal kidney HEK293 cells, and primary mouse kidney cells. When compared with our previous semiquantitative RT-PCR data of various cancer cell lines (Table 2), it is evident that the results from our enzymatic assays are in line with the results from our RT-PCR data. Further enzymatic assays will be carried out to compare arginase activities between the various prostate cancer cell lines as well as to assay for levels of ODC expression using another standard protocol (Brosnan *et al.* 1983).

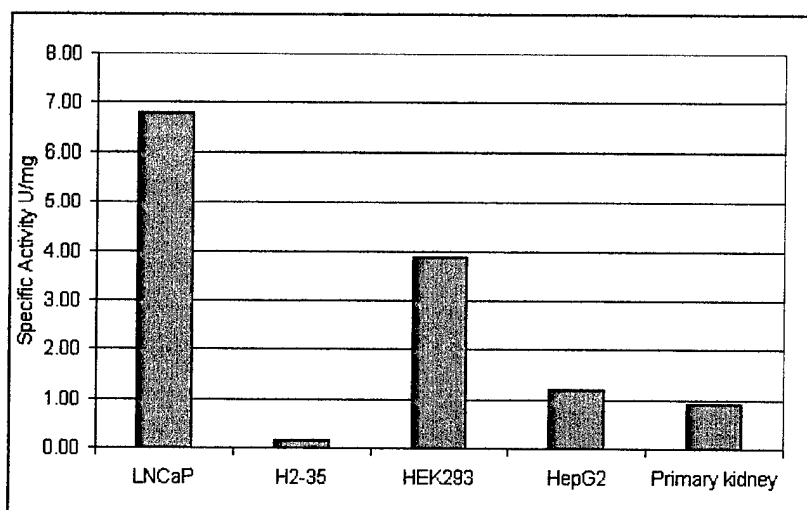


Figure 4. Arginase activity in native cell lines and mouse primary kidney cells.

Table 2. Semiquantitative RT-PCR Analysis for Expression of Selected Arginine and Polyamine Metabolic Genes.

Cell Line	Cancer Type	AI	AII	OAT	ODC	ADC	AGM	β -Actin
HepG2	Hepatoblastoma	1	3+	4	4	3+	3+	4+
HEK	Embryonic Kidney	0	3	4	4	2	2+	4+
Colo205	Colon	0	0	0	1+	2+	3+	4+
MiaPaCa2	Pancreas	0	3+	0	2+	0	1+	4+
PanC1	Pancreas	0	3	4	1+	2+	2+	4+
RecEp	Kidney	0	3+	2	4	0	0	4+
RecKr	Kidney	0	3	1	3+	0	0	4+
RecRu	Kidney	1	3+	1	2	3	0	4+
LnCap	Prostate	1+	4	3	4+	3	0	4+
SH-SY-5Y	Neuroblastoma	0	3+	0	0	2+	2	4+
NT2/D1	Embryonal Carcinoma	0	0	2	2	2+	2	3+

Task 2.c cDNA Miniarrays

We have had several trial attempts at constructing our own miniarrays containing spotted probes for the arginase-related genes of interest on nylon membranes, followed by radiolabeled mRNA hybridization and autoradiography. To date the hybridization signals have been suboptimal and inconsistent (not shown). We are working on modifications of the technique and, as an alternative, the use of commercially available microarrays.

Task 3.a Inhibitors of Gene Expression

Due to the increased interest and specificity of siRNA-mediated gene silencing technology (Lieberman *et al.* 2003), we have decided to employ these as our main tools for inhibiting gene expression instead of the earlier ribozyme technology we had shown and proposed in the grant application. To this end, we have already constructed a vector containing the promoter for the human U6 snRNA gene adjacent to a tetracycline-response element that has been used for cloning of our siRNAs (Figure 5). This vector was further modified recently through the insertion of a puromycin resistance gene for the selection of stable transfectants.

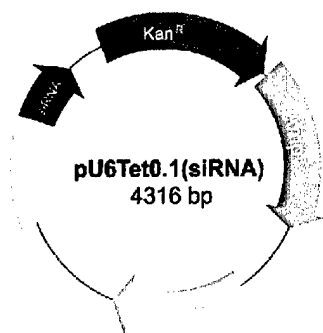


Figure 5. Our original siRNA cloning vector.

We have employed a unique strategy for the production of our siRNA vectors by inserting the targeting sequence through PCR amplification rather than traditional subcloning (Figure 6). Traditional subcloning can take extra time and has many more steps than a single PCR reaction. Our siRNA hairpin sequences were built into the PCR primers that were used to amplify the entire siRNA vector going in opposite directions. Thus, one only has to do an intramolecular ligation to produce an intact siRNA targeting vector. A list of the functional siRNA vectors constructed thus far can be seen in Table 3. Functionality of the individual siRNA sequences was tested through transfection into recipient cell lines and assaying changes in gene expression via RT-PCR (Figure 7). Vectors that were able to adequately silence gene expression would result in a reduction in band intensity as compared to that seen in the control cell line.

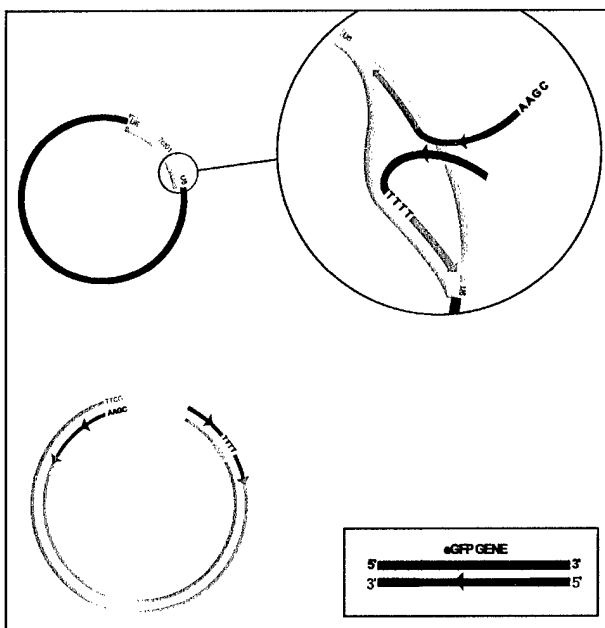


Figure 6. Insertion of a siRNA sequence into our siRNA targeting vector through long PCR.

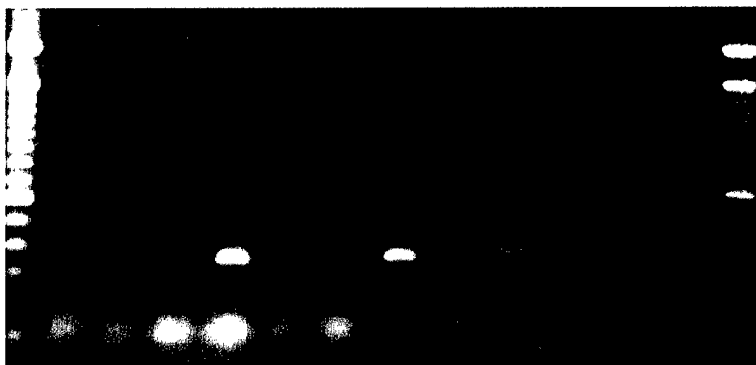


Figure 7. Assay for arginase II siRNA sequence validation by RT-PCR analysis.

SiRNA vectors were also validated by cotransfecting them with our overexpression constructs containing GFP fusions of the corresponding gene. The idea is that if a particular siRNA is able to target the mRNA of a particular gene and cause it to degrade, then fusing the gene to the green fluorescent protein gene should allow the effect to be visualized readily as a reduction in fluorescence intensity. As can be seen in Figure 8, cotransfection of several siRNA clones targeting human AI causes a reduction in fluorescence as compared to the cells transfected with the GFP fusion vector alone.

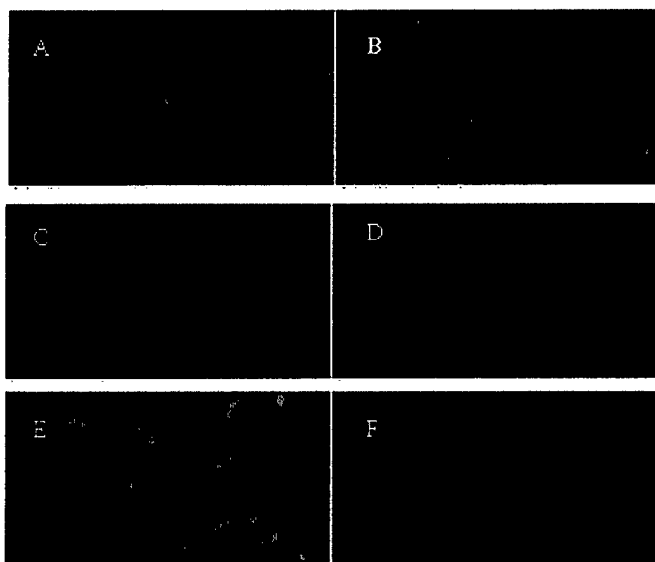


Figure 8. siRNA vector validation using fluorescence intensity. All transfections were done using HEK293 cells. (A,B) Transfection using human AI GFP fusion vector alone. (C,D,F) Transfection using human AI GFP fusion vector as well as three different siRNA clones targeting human AI. (E) Transfection with a control pLEGFP vector for transfection efficiency.

Task 3.b Inhibitors Using the Tetracycline-Controlled Transactivator

As stated above, our siRNA vectors also contain a tetracycline-responsive element adjacent to the U6 promoter. Therefore, levels of the siRNAs can be controlled in cell lines through addition of doxycycline to the medium. Prostate cell lines containing stable expression of various siRNA vectors are in development, and it will be of great interest to determine the consequences that silencing each gene will exert on the progression of prostate cancer. We already have prior experience utilizing tetracycline-responsive vectors for other purposes (see Task 5), and we are confident that we will be able to translate this technology toward our prostate cancer studies as well.

Task 3.c Growth Inhibition and Apoptosis

Nothing to report on this aim as of yet. We expect data in the near future.

Task 4.a Arginase Overexpression

As stated earlier, we have already constructed overexpression GFP fusion constructs for most of the various genes we are interested in studying. Each cDNA was subcloned into the pcDNA3.1/NT-GFP-TOPO vector (Invitrogen) to produce GFP fusions at the N-terminal end of the genes. Arginase II, however, was subcloned into the partner vector to create a GFP fusion at the C-terminal end of the gene. The utility of GFP as a visual marker for gene expression has been tested through transfection into various cell lines and observation using fluorescence microscopy (Figure 9). Furthermore, enzymatic assays have also been carried out on several of the vectors to ensure that a functional enzyme is being produced (Figure 10; Figure 11).

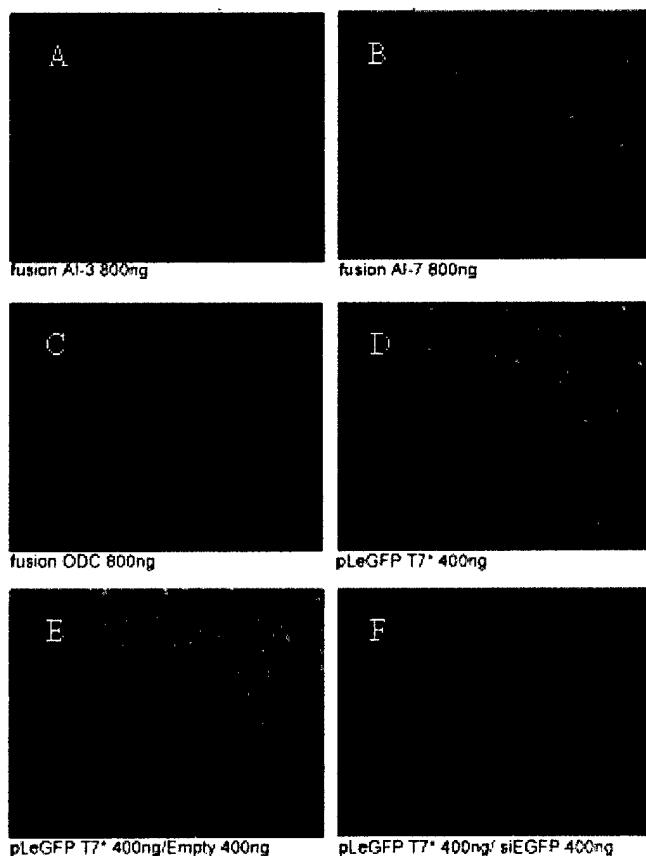


Figure 9. Fluorescence validation of GFP fusion proteins. All transfections were carried out using HEK293 cells. (A)(B) Transfection of two different clones of the human AI GFP fusion vector. (C) Transfection of the human ODC GFP fusion vector. (D) Transfection with a control pLEGFP vector for transfection efficiency. (E) Cotransfection with the pLEGFP control vector and an empty siRNA vector. (F) Cotransfection with the pLEGFP vector and an siRNA vector targeting the GFP gene sequence.

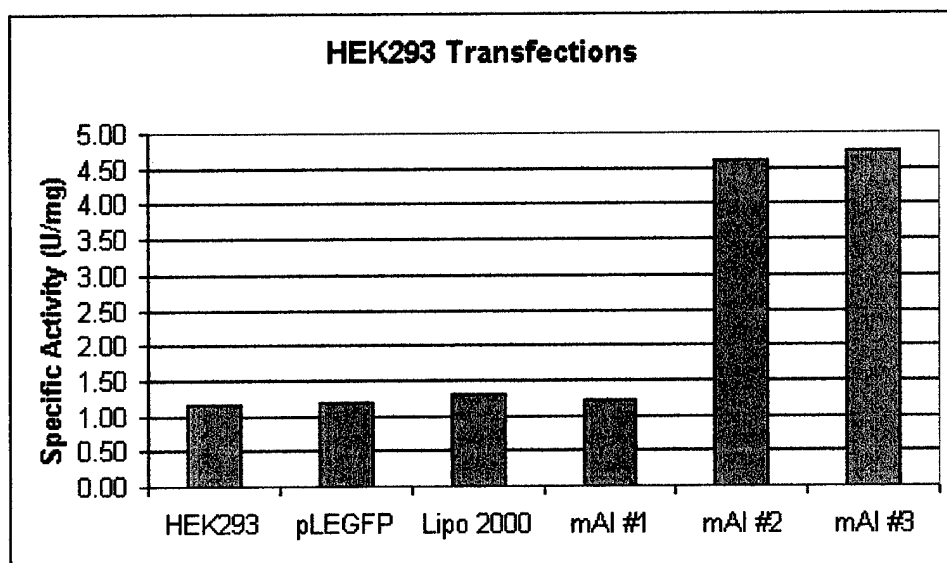


Figure 10. Enzymatic activity assays of AI GFP fusion vectors. As can be seen in this figure, clone #1 produces a protein with no arginase activity, whereas clones #2 and #3 produce functional fusion proteins. Untransfected cells (HEK293), empty vector (pLEGFP), and transfection reagent alone (Lipo 2000) were used as controls.

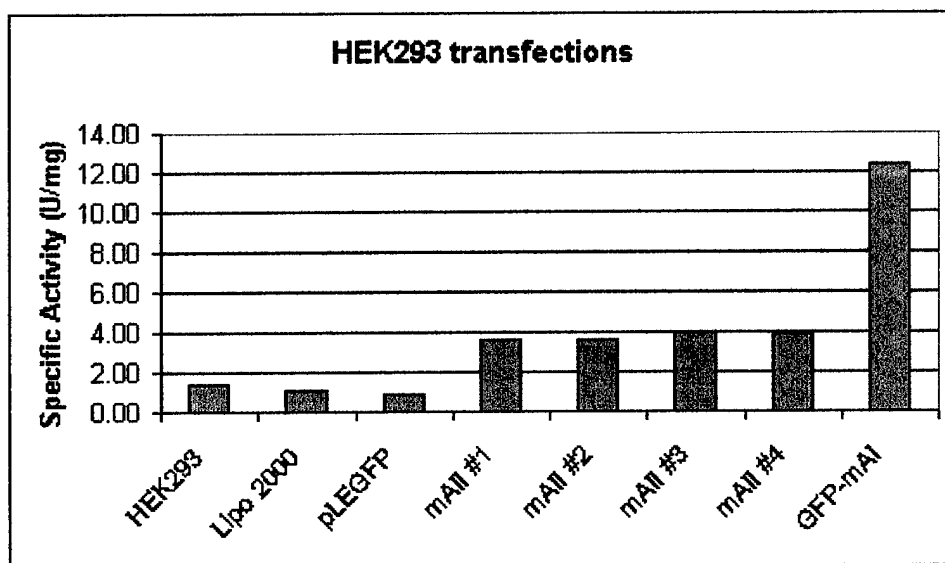
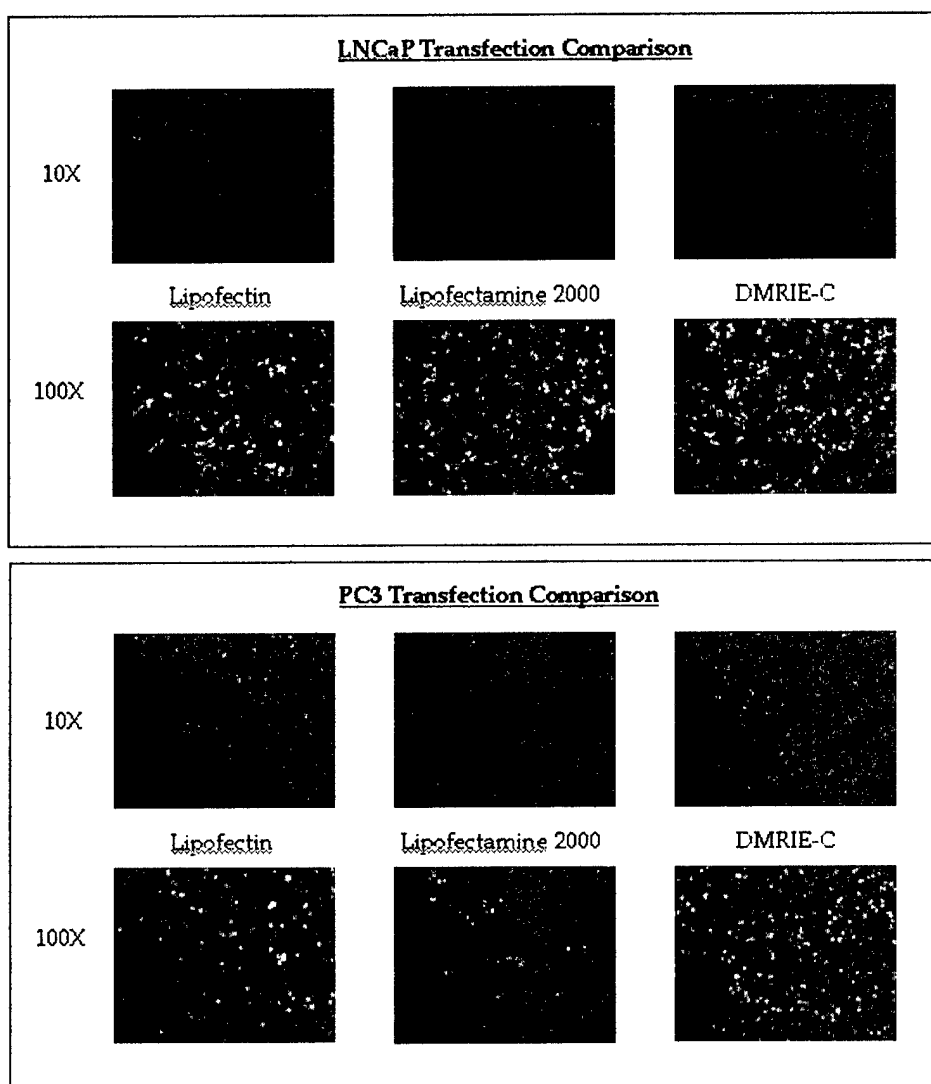


Figure 11. Enzymatic activity assays of AII GFP fusion vectors. As seen in this figure, all four mouse AII GFP fusion clones show arginase activity, though the AI GFP fusion arginase activity is noticeably higher. Untransfected cells (HEK293), empty vector (pLEGFP), and transfection reagent alone (Lipo 2000) were used as controls.

While our initial studies used HEK cells because of their reliable transfection efficiency, transfection conditions have also been tested and optimized for the three prostate cancer cell lines using a constitutively expressing GFP control plasmid (Figure 12), and the normal prostate line (PZ-HPV-7) will be evaluated in the near future. For the first round of stable transfections, we are using the LNCaP cell line and the AI and ODC overexpression constructs and respective siRNA vectors. All of the overexpression GFP fusions were originally cloned into a vector containing the neomycin resistance gene, which would have allowed for the creation of stable cell lines in a little more than a month. However, the decision was made to transfer all of these fusions to a new vector containing the resistance gene for blasticidin-S. Blasticidin-S is a much more highly potent antibiotic (Crivello & Jefcoate 1978), allowing for the creation of stable cell lines in one to two weeks.



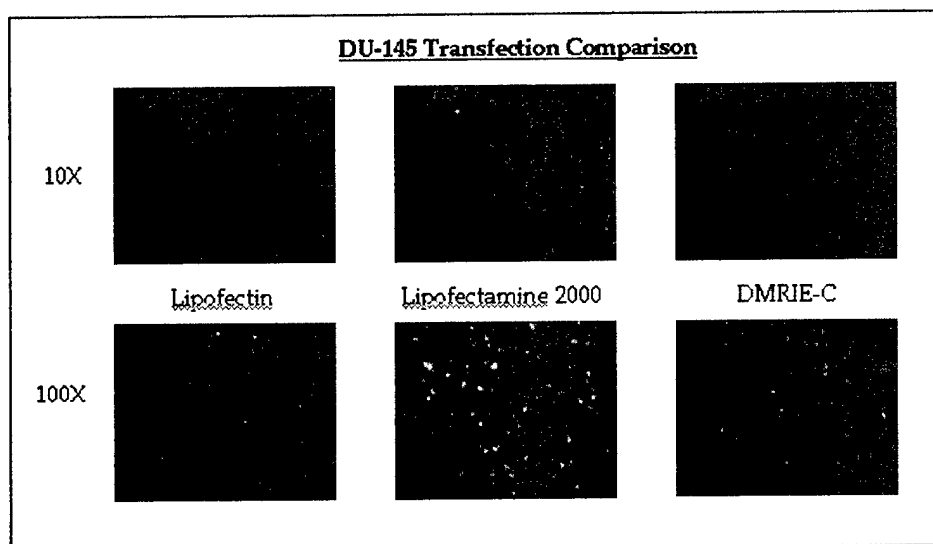


Figure 12 (continued from previous page). Transfection optimization of the LNCaP, PC-3, and DU-145 cell lines. For all studies thus far, the Lipofectamine 2000 reagent (Invitrogen) has been used.

For testing the efficacy of this system in tissue culture, we have also constructed a vector containing a GFP-arginase II fusion gene under the control of the tetracycline response element. As is, this vector could be transfected into any cell line with stable expression of the *Tet* regulator in order to provide level controllable expression of the transgene. To this end, we have also constructed another vector that will allow for the rapid selection of *tet*-regulatable cell lines using resistance to blasticidin-S. Besides the *Tet* regulator, the vector contains a blasticidin-GFP fusion gene that is flanked by *loxP* sites. The advantages of this vector are several-fold: the transfection efficiency can be evaluated readily by a visual check for GFP fluorescence; the *tet* regulator can be rapidly integrated into the cellular genome using blasticidin resistance; and once a stable cell line is created, the entire GFP fusion gene can be excised by adding *Cre* recombinase, resulting in the removal of everything between the two *loxP* sites (Sauer & Henderson 1989). The remainder of the *Tet* regulator vector can be maintained in the cell by using the neomycin resistance gene, which is also present on the plasmid. Finally, by virtue of its having been excised from the plasmid, resistance to blasticidin can be used to stably incorporate another vector into the same cell line such as the one with the *Tet* response element adjacent to the GFP-arginase II fusion protein. In this way, multiple cell lines exhibiting tetracycline control of the arginase II gene could be constructed in a relatively short period of time. Both of these vectors will be undergoing validation in the near future.

A listing of the various overexpression and inhibitory vector constructs successfully prepared during the first year of the project is presented below (Table 3). Pilot transfection experiments in the prostatic cell types listed above are now ongoing, primarily to establish optimal amounts and conditions to ensure adequate transfection efficiencies and target cell survival. In addition, we still have access to the hammerhead ribozyme constructs for AI, ODC, OAT and agmatinase which we had prepared at the

time of the initial grant proposal submission, but in the interim it has become clear, both by our results and those in the literature, that siRNA is a more effective and efficient means toward inhibition of specific gene expression (Lieberman *et al.* 2003; Lewin & Hauswirth 2001).

Table 3. Listing of GFP Fusion and siRNA Vectors Constructed During the First Year.

GFP Fusion Overexpression Constructs	siRNA Vectors
Human AI	Human AI
Mouse AI	Human AII
Human AII	Human ADC
Mouse AII	Human Agmatinase
Human Agmatinase	Human ODC
Mouse Agmatinase	Human OAT
Human ODC	
Human OAT	
Human ADC	
Mouse ADC	

Task 5 Nude Mice and Human Samples

As proposed for later in our project (Task 5), *ex vivo* cell models containing either overexpressing or inhibiting constructs of the above genes involved in polyamine synthesis will be transplanted into nude mice (Lee *et al.* 2003; Meyerrose *et al.* 2003) and evaluated for tumorigenicity. Experimental tumors will be compared with those formed from untransfected control cells on the basis of latency, growth rate, apoptosis, and metastases, as well as on a genetic basis by looking at changes in gene expression and polyamine levels. Experiments have not yet been started on this aim, as they were planned for late in Year 2 and Year 3; however, it is our hope that the results we obtain will correlate with those seen now in tissue culture studies and will provide further insight into the role of polyamine synthesis on prostate cancer progression. Our group already has extensive experience with mouse models, having earlier constructed a knockout strain for arginase I (Iyer *et al.* 2002), and this line, along with the AII knockout recently made available to us (see below), will be useful *in vivo* “reagents” for these studies.

Since the initial writing of the grant proposal, we have also acquired the TRAMP (transgenic adenocarcinoma mouse prostate) transgenic mouse line from the National Cancer Institute, a strain that was not previously available. These mice were derived using a minimal probasin promoter to drive expression of SV40 large T and small t tumor antigens, and the mice develop high-grade PIN (prostatic intraepithelial neoplasia) or prostate cancer within 12 weeks of age, followed by metastases by 30 weeks (for review, see Sharma and Schreiber-Agus 1999). We have just recently begun crossing our AI

heterozygous knockout mice into the TRAMP line in order to see if there are any alterations in prostate tumor onset or aggressiveness due to a lack of AI expression. Furthermore, as noted above, we were recently able to acquire the arginase II knockout mouse from our colleague William O'Brien at Baylor College of Medicine (Shi *et al.* 2001), and have thus far been using it for other research studies in Dr. Cederbaum's laboratory. However, we would eventually plan to breed the arginase II homozygote knockout mice into the TRAMP line to likewise look for any changes in tumor progression due to a lack of arginase II expression. Given our hypothesis that AII is the main isozyme involved in polyamine synthesis, we would predict this cross to be more revealing.

In the event that it becomes necessary to construct a transgenic mouse of our own at some point in the future, we have constructed and validated a vector with a probasin promoter upstream of the GFP gene. If it appears that our tissue culture and nude mouse model studies are supportive of our hypothesis, we would like to eventually derive a transgenic mouse with targeted expression of arginase II in the prostate. This would allow for study of the effects of AII on tumor growth without the need for subcutaneous transplantation of transfected cells into a nude mouse as proposed for Task 5. Since tight control of AII expression would be more desirable than having it occur constitutively, an AII transgenic mouse could also incorporate the tetracycline controllable system (Knott *et al.* 2002). This would enable us to decrease the level of arginase II expression in the prostate simply by adding tetracycline to the drinking water of the mice. We have already utilized this system for the liver-specific transgenic rescue of our AI knockout mice and believe it would be highly valuable for such studies on the second isoform of arginase as well (Figure 13; Figure 14). While some of these studies go beyond what was proposed in the original grant application, the intent is to maximize our efforts by taking advantage of related *in vivo* models being developed and studied in other sections of our and Dr. Cederbaum's laboratories.

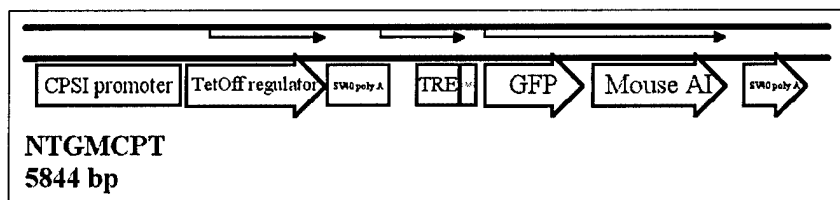


Figure 13. Map of vector used to create our AI transgenic mouse. This vector utilizes tetracycline-regulated expression of our transgene under a tissue specific promoter.

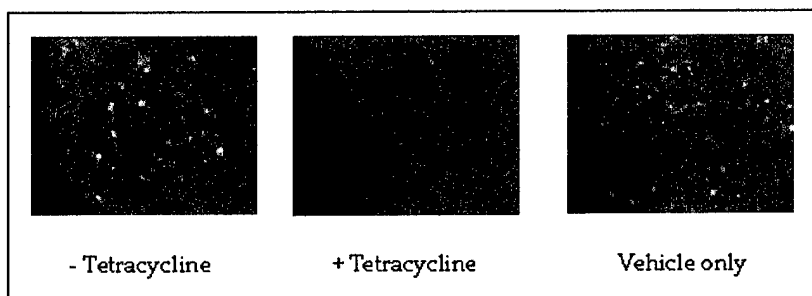


Figure 14. Validation of tetracycline responsiveness using our AI transgenic vector. This vector was transfected into HEK293 cells and the cells were observed 48 hours later using fluorescence microscopy.

Key Research Accomplishments

- Establishment of necessary cell lines representing the range of prostatic tissue neoplasia and differentiation, from essentially normal to poorly differentiated/invasive
- Construction of vectors containing regulatory elements for effecting the expression or silencing of both of the arginase isozymes and all of the associated genes of arginine metabolism of interest to us
- Optimization of transfection conditions for the various cell lines and vector constructs
- Receipt and initial breeding of the TRAMP mouse to be used as an *in vivo* model of prostatic tumor formation that can be manipulated by the transgenic constructs mentioned above and by crossing with our arginase knockout mouse strains

Reportable Outcomes

Nothing to report at this time. We expect abstracts and other such outcomes to emerge in Year 2.

Conclusions

We consider the accomplishments of Year 1 reported here to represent the necessary groundwork for the more exciting studies to be pursued in Years 2 and 3. As described in our grant proposal, it was necessary to have most of the required molecular reagents and animal strains in hand and well characterized before we could embark on meaningful experimental studies. As such, we feel we are now in possession of a set of constructs, possibly unique in the world, for dissecting the arginine metabolic pathway as it relates to the pathogenesis of prostate cancer.

Despite the high incidence of prostate cancer, relatively little is known about the biochemical and molecular mechanisms controlling benign and malignant prostatic growth. This project sets out a novel and original program that seeks to elucidate fundamental underlying mechanisms linking our surprising observation of elevated prostatic arginase AII levels with promotion and potential inhibition of cancer of the prostate. We are building upon our group's long track record of arginase research in the context of a metabolic disorder, arginase deficiency, and are now applying these resources for the first time to an investigation of what we believe to be this enzyme's fundamental involvement in prostate cancer cell growth. This hypothesis is based on the locus of arginase activity at the convergence of critical urea cycle, polyamine synthetic, and nitric oxide pathways, all of which are key aspects of prostate physiology and cell proliferation. We believe this work will enhance our understanding of the fundamental mechanisms of prostatic neoplasia and also suggest new and specific molecular targets for both diagnosis and therapy.

References

- Brosnan ME, Farrell R, Wilansky H, Williamson DH. 1983. Effect of starvation and refeeding on polyamine concentrations and ornithine decarboxylase antizyme in mammary gland of lactating rats. *Biochem J.* 212(1):149-53.
- Crivello JF, Jefcoate CR. 1978. Mechanisms of corticotropin action in rat adrenal cells. I. The effects of inhibitors of protein synthesis and of microfilament formation on corticosterone synthesis. *Biochim Biophys Acta* 542:315-329.
- Dorhout B, Kingma AW, de Hoog E, Muskiet FA. 1997. Simultaneous determination of polyamines, N-acetylated polyamines and the polyamine analogues BE-3-3-3 and BE-4-4-4-4 by capillary gas chromatography with nitrogen-phosphorus detection. *J Chromatogr B Biomed Sci Appl* 700(1-2):23-30.
- Iyer RK, Yoo PK, Kern RM, Rozengurt N, Tsoa R, O'Brien WE, Yu H, Grody WW, Cederbaum SD. 2002. A mouse model for human arginase deficiency. *Molec Cell Biol* 22:4491-4498.
- Knott A, Garke K, Urlinger S, Guthmann J, Muller Y, Thellmann M, Hillen W. 2002. Tetracycline-dependent gene regulation: Combinations of transregulators yield a variety of expression windows. *BioTechniques* 32:796-806.
- Lee C, Shevrin DH, Kozlowski JM. 1993. In vivo and in vitro approaches to study metastasis in human prostatic cancer. *Cancer Metast Rev* 12:21-28.
- Lewin AS, Hauswirth WW. 2001. Ribozyme gene therapy: applications for molecular medicine. *Trends Mol Med.* 7(5):221-8.
- Lieberman J, Song E, Lee S-K, Shankar P. 2003. Interfering with disease: Opportunities and roadblocks to harnessing RNA interference. *Trends Molec Med* 9:397-403.
- Meyerrose TE, Herrbrich P, Hess DA, Nolte JA. 2003. Immune-deficient mouse models for analysis of human stem cells. *BioTechniques* 35:1262-1272.
- Morris SM Jr. 2002. Regulation of enzymes of the urea cycle and arginine metabolism. *Annu Rev Nutr* 22:87-105.
- Sauer B, Henderson N. 1989. Cre-stimulated recombination at loxP-containing DNA sequences placed into the mammalian genome. *Nucl Acids Res* 17:147-161.
- Sharma P, Schreiber-Agus N. 1999. Mouse models of prostate cancer. *Oncogene* 18:5349-5355.

Shi O, Morris SM Jr, Zoghbi H, Porter CW, O'Brien WE. 2001. Generation of a mouse model for arginase II deficiency by targeted disruption of the arginase II gene. *Mol Cell Biol* 21:811-813.

Spector EB, Kiernan M, Bernard B, Cederbaum SD. 1980. Properties of fetal and adult red blood cell arginase: a possible prenatal diagnostic test for arginase deficiency. *Am J Hum Genet* 32(1):79-87.

Yu H, Yoo PK, Aguirre CC, Tsoa RW, Kern RM, Grody WW, Cederbaum SD, Iyer RK. 2003. Widespread expression of arginase I in mouse tissues: Biochemical and physiological implications. *J Histochem Cytochem* 51:1151-1160.